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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	Attorney's Docket Number 045636-5037	U.S. Application No. (If Assigned) 09/582771
International Application. No.	International Filing Date	Priority Date Claimed
PCT/FR98/02899	29 December 1998	30 December 1997

Title of Invention

PEPTIDE EPITOPES RECOGNIZED BY ANTI-FILAGGRIN AUTO-ANTIBODIES IN SERUM FROM RHEUMATOID ARTHRITIS PATIENTS

Applicants For DO/EO/US

Guy SERRE, Elisabeth GIRBAL-NEUHAUSER, Christian VINCENT, Michel SIMON, Mireille SEBBAG, Pascal DALBON, Colette JOLIVET-REYNAUD and Michel ARNAUD

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A Verified Statement Claiming Small Entity Status
16. [X] Other items or information:
    a. [X] Copy of Form PCT/IB/304
    b. [X] Copy of Form PCT/IB/308
    c. [X] WIPO Publication WO 99/35167

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
Unassigned 09/582711	PCT/FR98/02899	045636-5037

17. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$840.00
International preliminary examination fee paid to
USPTO (37 CFR 1.482).....$670.00
No international preliminary examination fee paid to
USPTO (37 CFR 1.482) but international search fee
paid to USPTO (37 CFR 1.445(a)(2)).....$760.00
Neither international preliminary examination fee
(37 CFR 1.482) nor international search fee
(37 CFR 1.445(a)(2)) paid to USPTO.....$970.00
International preliminary examination fee paid to USPTO
(37 CFR 1.482) and all claims satisfied provisions
of PCT Article 33(2)-(4).....$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $ 840.00

Surcharge of $130.00 for furnishing the oath or declaration later than **[ ] 20 [ ] 30** months from the earliest claimed priority date (37 CFR 1.492(e)). $

Claims	Number Filed	Number Extra	Rate	
Total Claims	11- 20 =	0	X $18.00	$0.00
Independent Claims	1 - 3 =	0	X $78.00	$
Multiple dependent claim(s) (if applicable)			+$260.00	$0.00

TOTAL OF ABOVE CALCULATIONS = **$840.00**

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28) -$

SUBTOTAL = **$840.00**

Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +$

TOTAL NATIONAL FEE = **$840.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property $0.00

TOTAL FEES ENCLOSED = **$840.00**

Amount to be refunded $
charged $

a. [X] A check in the amount of **$840.00** to cover the Basic fee is enclosed.

b. [ ] Please charge my Deposit Account No. 50-0310 in the amount of $0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] **Except** for issue fees payable under 37 C.F.R. §1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 CFR §1.16 and §1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

SEND ALL CORRESPONDENCE TO:
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Submitted: June 30, 2000

PATENT
ATTORNEY DOCKET NO. 045636-5037

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : SERRE et al.	
U.S. Application No.: To Be Assigned	Group Art Unit: Unassigned
Date of National Stage Entry : June 30, 2000	Examiner: Unassigned
Based on PCT/FR98/02899 Filed : December 29, 1998	
For: PEPTIDE EPITOPES RECOGNIZED BY ANTI- FILAGGRIN AUTOANTIBODIES IN SERUM FROM RHEUMATOID ARTHRITIS PATIENTS	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application on the merits, please amend the application, without prejudice, as follows:

ABSTRACT: Please add the abstract provided on a separate sheet.

IN THE CLAIMS:

Please cancel claim 4.

Please amend claims 1-3 and 5-7 as follows:

Claim 1, line 1, change "Peptide" to -- A peptide--;

Claim 1, line 3, change "characterized in that" to -- wherein --;

Claim 1, line 4, change "centred" to -- centered --;

Claim 2, line 1, change "Peptide" to -- The peptide--; and,

change "characterized in" to -- wherein --;

Claim 2, line 2, delete "that it comprises";

after "motif" add -- comprises --; and,

after "Ser-Cit-His" add a comma -- , --;

Claim 3, line 1, change "Artificial" to -- An artificial --;

Claim 3, line 3, delete "characterized in that it";

Claim 3, line 4, change "comprises" to -- comprising --; and

delete "or consists of";

Claim 3, line 5, change "either of Claims 1 and 2" to -- Claim 1 --;

Claim 5, line 1, change "Antigenic" to -- An antigenic --;

Claim 5, line 3, delete "characterized in that it";

Claim 5, line 4, change "contains" to -- comprising --; and,

delete "any one of";

Claim 5, line 5, change "Claims" to -- Claim --; and,

delete the remainder of the line, "to 3 which is optionally labelled with and/or";

Claim 5, line 6, delete the entire line except the period, "conjugated to a carrier molecule";

Claim 6, line 1, change "Method" to -- A method --;

Claim 6, line 2, delete "this";

Claim 6, line 3, delete "method being characterized in that it"; and,

change "comprises" to -- comprising --;

Claim 6, line 5, change "any one of Claims 1 to 3" to -- Claim 1 --;

Claim 6, line 7, change "the" to -- any --;

Claim 6, line 8, delete "possibly" ; and,

after "present" add -- in the biological sample --;

Claim 6, line 9, change "the" to -- any --;

Claim 6, line 10, delete "possibly" ;

Claim 7, line 1, change "Kit" to -- A kit --;

Claim 7, line 2, delete "characterized in";

Claim 7, line 3, change "that it comprises" to -- comprising --; and,

delete "any";

Claim 7, line 4, change "one of Claims 1 to 3" to -- Claim 1 --;

Claim 7, lines 7 and 8, delete in their entirety except the period, "and/or means for detecting said antigen/antibody complex".

Please add the following claims 8-12.

8. The artificial antigen of Claim 3 wherein the antigen consists of at least one peptide comprising a tripeptide motif centered on a citrulline residue, which is specifically present on at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6.

9. The antigenic composition of Claim 5 wherein the at least one antigen is labeled.

10. The antigenic composition of Claim 5 wherein the at least one antigen is conjugated to a carrier molecule.

11. The kit of Claim 7 further comprising means for detecting the antigen/antibody complex.

12. A kit for detecting rheumatoid arthritis-specific autoantibodies in a biological sample, comprising at least one antigen according to Claim 1, as well as means for detecting the antigen/antibody complex.

REMARKS

The changes to the claims requested above have been made so as to eliminate multiple claim dependencies and to present claim language more conventional for practice in the United States. These changes do not introduce new matter, nor do they alter the subject matter presented and examined in the corresponding International Application.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

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ABSTRACT OF THE DISCLOSURE

The invention concerns peptides comprising epitopes recognized by antifilaggrin in serum from rheumatoid arthritis patients. Said epitopes comprising a tripeptide motif centered on a citrulline residue. The invention also concerns artificial antigens comprising said epitopes, and their use for diagnosing rheumatoid arthritis.

PEPTIDE EPITOPES RECOGNIZED BY ANTI-FILAGGRIN AUTO-ANTIBODIES IN SERUM FROM RHEUMATOID ARTHRITIS PATIENTS

The present invention relates to novel preparations of antigens which are specifically recognized by rheumatoid arthritis-specific autoantibodies.

Rheumatoid arthritis (hereafter abbreviated to "RA") is the most frequent of the chronic inflammatory rheumatisms. It is an autoimmune disease, and the serum from affected patients contains autoantibodies, certain of which are specific, and may constitute a marker for this disease, allowing its diagnosis even at early stages. Research has thus been carried out with a view to identifying antigens recognized by these antibodies, in order to obtain purified preparations thereof which can be used in conventional techniques of immunological diagnosis.

Autoantibodies which are specifically present in RA patients, and which react with a rat oesophagial epithelial antigen, were described for the first time by B.J.J. Young et al. in Br. Med. J. 2:97-99, (1979). These autoantibodies were, at the time, named "anti-keratin antibodies".

During previous work, the inventors' team obtained, from human and murine malpighian epithelia, preparations of antigens related to filaggrin and to profilaggrin which are recognized specifically by antibodies present in serum from rheumatoid arthritis patients, and showed that the "anti-keratin antibodies" were in fact anti-filaggrin autoantibodies (hereafter named "AFA"). Application EP 0 511 116 describes these antigenic preparations and their use for diagnosing rheumatoid arthritis.

Filaggrins are a family of proteins which have been identified in various species, inter alia in humans, rats, mice and guinea pigs, in keratinizing malpighian epithelia [for a review on filaggrins, cf. Dale et al. [The Keratinocyte Handbook, Cambridge

University Press, pp. 323-350, (1994)]]. They are derived from the dephosphorylation and proteolysis of a precursor, profilaggrin, which consists essentially of repeated filaggrin domains separated by interdomain peptide segments.

The gene encoding profilaggrin is composed of repeated subunits, each of which encodes a filaggrin molecule, separated by portions encoding the inter-domain peptide segments. All the repeat units encoding each of the human filaggrins have the same length (972 base pairs in humans); however, in humans, considerable sequence variation (10-15%) is observed from one subunit to the other. While most are conservative, some of these variations induce amino acid changes and, in certain cases, changes in the electrical charge of the protein. Thus, human filaggrins form, independently of post-transcriptional modifications, a heterogeneous population of molecules of similar size, but of different sequences and charges (pHi equal to 8.3 ± 1.1) [Gan et al., Biochem. 29, pp. 9432-9440 (1990)].

Profilaggrin is a protein of high molecular weight (approximately 400,000 in humans) which is soluble in the presence of high concentrations of salts or of urea. It possesses a high content of basic amino acids (arginine and histidine) and of glycine, serine and glutamic acid. It is low in nonpolar amino acids, and contains neither methionine, nor cysteine, nor tryptophan. It is highly phosphorylated on serine residues, which gives it an isoelectric point close to neutrality.

Profilaggrin is cleaved into filaggrin units during a complex maturation process which involves dephosphorylation, followed by cleavage by proteases at the interdomain segments. This cleavage generates, first of all, fragments of intermediate size, and then the functional filaggrin molecules.

Filaggrins derived from the dephosphorylation and cleavage of profilaggrin are basic proteins, the amino acid content of which is similar to that of

profilaggrins. They participate in the organization of keratin filaments, and undergo a progressive maturation during which the arginine residues, which are basic, are converted into citrulline residues, which are neutral, under the action of peptidylarginine deiminase [Harding C.R. and Scott I.R., J. Mol. Biol. 170, pp. 651-673 (1983)]. This leads to a reduction in their affinity for keratins, from which they detach; they are then totally degraded under the action of various proteases.

Filaggrin and profilaggrin properties have been particularly well studied in rats, in mice and in humans. The size of profilaggrin varies, according to the species, from 300 to 400 kD, and that of filaggrins from 27 to 64 kD.

The polymorphism observed in humans between the sequences of filaggrin units within the same profilaggrin gene does not appear in rats and mice. Filaggrins also exhibit great inter- and intraspecific variability in their sequence. This variability does not however affect their functional properties, or their overall amino acid composition, or their biochemical properties. Similarly, the tissue localizations of profilaggrin and of filaggrins are identical in the various mammals studied.

In continuing their work, the inventors noticed that profilaggrin present in keratohyalin granules of human epidermis was not recognized, unlike filaggrins, by AFAs [Simon et al. Clin. Exp. Immunol. 100, 90-98 (1995)]. They then tested the reactivity of AFAs with recombinant filaggrin, and observed that this was not recognized either. Conversely, it had previously been observed that the forms of human epidermal filaggrin principally recognized by AFAs were the acido-neutral forms described by Simon et al. [J. Clin. Invest., 92, 1387, (1993)], and in Application EP 0 511 116. The fact that these acido-neutral forms correspond to a late maturation stage of filaggrin made it possible to presume that all or part of the post-translational

modifications which intervene up to this stage are involved in the formation of the epitopes recognized by AFAs.

To verify this hypothesis, the inventors attempted to reproduce these post-translational modifications *in vitro*, using recombinant filaggrin, in order to determine which are capable of influencing filaggrin antigenicity.

They thus in fact observed that citrullination of filaggrin was enough to generate epitopes recognized by AFAs. Specifically, they observed, by carrying out *in vitro* deimination of recombinant filaggrin, that replacing at least one portion of the arginines with citrullines allows the production of an antigen which is recognized specifically by AFAs present in serum from RA patients. They also located regions which, after citrullination, were highly immunoreactive with respect to anti-filaggrin autoantibodies. They are in particular the region corresponding to the C-terminal portion (amino acids 144 to 324), and in particular to amino acids 144 to 314, as well as the region corresponding to amino acids 76 to 144 and the region corresponding to amino acids 71 to 119, of a human filaggrin unit. This work resulted in the production of artificial antigens, which are recognized specifically by AFAs present in serum from RA patients, and which consist of recombinant or synthetic polypeptides derived from the sequence of filaggrin or from portions of it, by substituting at least one arginine residue with a citrulline residue. These antigens, as well as their use, form the subject of Application FR 96/10651, filed on 30 August 1996 in the name of Biomérieux.

In continuing their work, the inventors managed to select, using the sequence of one filaggrin unit, peptides in which substituting at least one arginine residue with a citrulline residue gives rise to epitopes which are recognized specifically by AFAs present in serum from RA patients.

The sequences of these peptides are identified

in the attached sequence listing under the numbers SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 6.

"Filaggrin unit" is intended to mean a polypeptide, the sequence of which is that of the translation product of any one of the subunits encoding a filaggrin domain of the human, or any other species, profilaggrin gene, or is a consensus sequence, this theoretical sequence being obtained from filaggrin domain sequences.

The inventors have now identified epitopes recognized by anti-filaggrin autoantibodies: these epitopes comprise a tripeptide motif centred on a citrulline residue, which is specifically present on the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6, and which is absent from the sequence SEQ ID NO: 4.

It is in particular the tripeptide motif Ser-Cit-His in which Cit represents a citrulline residue.

A subject of the present invention is a peptide which constitutes an epitope recognized by anti-filaggrin autoantibodies present in serum from rheumatoid arthritis patients, characterized in that said epitope comprises a tripeptide motif centred on a citrulline residue, which is specifically present on at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6.

According to a preferred embodiment of the present invention, said peptide comprises at least one pentapeptide motif centred on a citrulline residue, which is present on at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6.

Advantageously, said peptide comprises the tripeptide motif Ser-Cit-His in which Cit represents a citrulline residue.

By way of example, mention will be made of peptides derived, by citrullination, from peptides

which comprise the pentapeptide motif X1-Ser-Arg-His-X2 in which X1 = Ser or Gly, and X2 = Ser or Pro, and among them, peptides which comprise the hexapeptide motif X0-X1-Ser-Arg-His-X2 or the heptapeptide motif X0-X1-Ser-Arg-His-X2-X3, in which X1 and X2 are as defined above, X0 = Asp and X3 = Gly or Arg.

The peptides in accordance with the invention allow the preparation of artificial antigens which are recognized specifically by anti-filaggrin autoantibodies present in serum from rheumatoid arthritis patients. These artificial antigens also form part of the subject of the present invention.

Artificial antigens in accordance with the invention comprise at least one peptide epitope centred on a citrulline residue as defined above. They consist for example of peptides of at least 5 amino acids, preferably at least 10 amino acids, and advantageously at least 14 amino acids. They can be peptides consisting of at least one fragment of at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6, or containing at least one such fragment. These peptides can comprise several citrullinated epitopes which are specifically recognized by AFAs, and which are of identical or different sequences.

The term "peptide" as used in the present application means in particular protein or protein fragment, or oligopeptide, which is extracted, separated or substantially isolated or synthesized, in particular those obtained by chemical synthesis or by expression in a recombinant organism; any peptide in the sequence of which one or more amino acids of the L series are replaced with an amino acid of the D series, and vice versa; any peptide in which one at least of the CO-NH bonds, and advantageously all the CO-NH bonds, of the peptide chain is (are) replaced with one or more NH-CO bonds; any peptide in which one at least of the CO-NH bonds, and advantageously all the CO-NH bonds, is or are replaced with one or more NH-CO

bonds, the chirality of each aminoacyl residue, whether or not it is involved in one or more abovementioned CO-NH bonds, being either conserved or inverted with respect to the aminoacyl residues constituting a reference peptide, these compounds also being referred to as immunoretroids, a mimotope, etc.

Antigens in accordance with the invention can for example be obtained by reacting PAD (peptidyl-arginine deiminase) with natural, recombinant or synthetic proteins or peptides comprising arginine residues and in particular comprising at least one arginine residue constituting the centre of a tri-peptide motif identical to those present in the sequences SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 6; they can also be obtained by peptide synthesis, by directly incorporating one or more citrulline residues, and preferably one or more epitopes comprising a citrulline residue as defined above, into the synthesized peptide.

A subject of the present invention is also the use of the antigens in accordance with the invention as defined above for diagnosing RA *in vitro*.

The present invention encompasses in particular antigenic compositions for diagnosing the presence of RA-specific autoantibodies in a biological sample, these compositions being characterized in that they contain at least one antigen in accordance with the invention which is optionally labelled with and/or conjugated to a carrier molecule.

A subject of the present invention is also a method for detecting RA-specific class G autoantibodies in a biological sample, this method being characterized in that it comprises:

- bringing said biological sample into contact with at least one antigen in accordance with the invention as defined above under conditions which allow the formation of an antigen/antibody complex with the RA-specific autoantibodies possibly present;
- detecting, by any suitable means, the antigen/anti-

body complex possibly formed.

This detection method can be implemented using a kit comprising at least one antigen according to the invention, as well as suitable buffers and reagents for constituting a reaction medium which allows the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

Said kit can also comprise, where appropriate, reference samples such as one or more negative serum (sera) and one or more positive serum (sera).

The present invention will be better understood with the aid of the further description which will follow, which refers to examples of preparation and of use of antigens in accordance with the invention.

EXAMPLE 1: *IN VITRO* DEIMINATION OF RECOMBINANT FILAGGRIN WITH PEPTIDYLARGININE DEIMINASE (P.A.D.)

Recombinant filaggrin is produced according to the following protocol:

A DNA fragment encoding a filaggrin unit is amplified from human genomic DNA (RAJI cells: ATCC CCL86) by PCR using the 2 primers below:

5' primer:

5' TTCCTATACCAGGTGAGCACTCAT 3'

3' primer:

5' AGACCCTGAACGTCCAGACCGTCCC 3'

The amplification product is cloned into the SmaI site of the vector pUC19. The recombinant clones are selected by verifying the presence of a 972-bp insert obtained after digestion with SacI and XbaI. This insert is then subcloned into pUC19. The insert resulting from this subcloning is then transferred into the vector pGEX (sold by the company Pharmacia), between the EcoRI and HindIII sites. In *E. coli*, the expression vector thus obtained expresses filaggrin in fusion with glutathione-S-transferase (GST) under the control of the Tac prokaryotic promoter. Recombinant protein synthesis is induced by adding isopropyl-β-D-galactoside (IPTG) to the culture.

The recombinant filaggrin thus obtained will be named hereafter: "fil-gst".

After electrophoresis, the existence of 9 fragments is observed, which result from a post-translational proteolysis of whole filaggrin.

The mixture of the 9 fragments is subjected to an *in vitro* deimination with peptidylarginine deiminase.

A preparation of rabbit muscle peptidylarginine deiminase (681 U/ml), sold by Takara Biomed Europe, is used according to the protocol recommended by the manufacturer.

The procedure conditions are as follows:

- reaction medium: 0.1 M Tris-HCl, 10 mM $CaCl_2$, 5 mM DTT, pH 7.4;
- enzyme/substrate ratio: 140 mU/µmol of filaggrin containing 10% of arginine, i.e. 4 mU/µmol of arginine;
- incubation: between 0 and 60 min at 50°C;
- termination of the reaction: heating for 3 min in Laemmli buffer.

The 8 reactions below are carried out in parallel.

(1) BSA (bovine serum albumin) incubated in reaction medium (1 h, 50°C) without P.A.D.

(2) BSA incubated in reaction medium (1 h, 50°C) with 60 mU of P.A.D.

(3) fil-gst incubated in reaction medium (1 h, 50°C) without P.A.D.

(4) fil-gst incubated in reaction medium (5 minutes at 50°C) with 60 mU of P.A.D.

(5) fil-gst incubated in reaction medium (15 minutes at 50°C) with 60 mU of P.A.D.

(6) fil-gst incubated in reaction medium (30 minutes at 50°C) with 60 mU of P.A.D.

(7) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of P.A.D.

(8) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of P.A.D. and in the presence of

10 mM of N-ethylmaleimide (inhibitor of P.A.D.).

1 µl of each sample is loaded on an electrophoresis gel (12.5% SDS-PHAST® gel, Pharmacia), and electrophoresis is carried out using the PHAST-SYSTEM® apparatus (Pharmacia) under the conditions recommended by the manufacturer. After transfer onto nitrocellulose, detection is carried out either with a pool of 5 sera from RA patients, diluted to 1/2000, or with the anti-filaggrin monoclonal antibody AHF2 [Simon et al. J. Invest. Dermatol. 105, 432, (1995)] at the concentration of 0.2 µg/ml.

The antigen/antibody complex is detected by the ECL technique using a peroxidase-coupled secondary antibody.

The results show that, in the absence of citrullination reaction, the fil-gst is not recognized by sera from RA patients, whereas right from 5 minutes of citrullination, it is detected by these sera. An increase in the reactivity with the pool of sera is observed when P.A.D. is reacted for 60 minutes at 50°C.

In addition, these results make it possible to presume that one or more epitopes of high affinity exist in the COOH-terminal moiety of filaggrin (positions 144 to 324), this or these epitope(s) being repeated between positions 76 and 144.

EXAMPLE 2: CITRULLINATION OF PEPTIDES S-47-S AND S-35-R WITH P.A.D., AND CITRULLINATED PEPTIDE REACTIVITY ASSAY

The 49-amino acid peptide S-47-S of sequence (1-letter code):

NH_2-STGHSGSQHSHTTTQGRSDASRGSSGSRSTSRETRDQEQSGDGSRHSGS-COOH

corresponding to amino acids 71 to 119 of the sequence of a human filaggrin unit and comprising 6 arginine residues, and

the 37-amino acid peptide S-35-R of sequence (1-letter code):

NH_2-SQDRDSQAQSEDSERRSASASRNHRGSAQEQSRDGSR-COOH

corresponding to amino acids 155 to 191 of the sequence of a human filaggrin unit and comprising 7

arginine residues were prepared by peptide synthesis. The peptides S-47-S and S-35-R are represented in the attached sequence listing under the respective numbers SEQ ID No: 3 and SEQ ID No: 4.

These 2 peptides, as well as fil-gst, were citrullinated by reacting P.A.D. for 30 minutes at 50°C in the same reaction medium as that indicated in Example 1. The conditions specific for each peptide and for fil-gst are as follows:

- peptide S-47-S: 4 mU/µmol arginine
- peptide S-35-R: 2.7 mU/µmol arginine
- fil-gst: as indicated in Example 1.

The reactivity of each peptide and that of fil-gst, before and after reacting the enzyme, with respect to the monoclonal antibody AHF4 and to the serum from an RA patient are compared by "dot-blot".

The procedure conditions are as follows:

- 0.5 µg per deposit of each antigen (peptides, fil-gst, acido-neutral variants of filaggrin (AVF)).
- Treatment of the nitrocellulose for 45 minutes at 80°C, before immunodetection.
- RA serum used at the dilution of 1/2000; monoclonal antibody AHF4 used at a concentration of 0.2 µg/ml.

The results show that:

- AHF4 recognizes citrullinated or noncitrullinated peptide S-47-S and fil-gst, but does not recognize citrullinated or noncitrullinated S-35-R.
- S-47-S is recognized, after citrullination, by the serum of the RA patient, whereas neither citrullinated nor noncitrullinated S-35-R is recognized. The same serum recognizes moreover citrullinated fil-gst and AVFs, but does not recognize noncitrullinated fil-gst.

EXAMPLE 3: SYNTHESIS OF CITRULLINATED AND NON-CITRULLINATED PEPTIDES E-12-H AND E-12-D, AND PEPTIDE REACTIVITY ASSAY

The peptides E-12-H and E-12-D were determined by reference to the nucleotide sequences of the human profilaggrin gene described by Gan S.Q. et al. [Biochemistry, 29: 9432-9440, (1990)].

The 14-amino acid peptide E-12-H of sequence (1-letter code):

NH_2-EQSADSSRHSGSGH-COOH

comprises 1 arginine residue, and

the 14-amino acid peptide E-12-D of sequence (1-letter code):

NH_2-ESSRDGSRHPRSHD-COOH

comprises 3 arginine residues.

The peptides E-12-H and E-12-D are represented in the attached sequence listing under the respective numbers SEQ ID No: 5 and SEQ ID No: 6.

These peptides were prepared by solid phase peptide synthesis.

The citrullinated peptides E-12-H and E-12-D were synthesized directly by incorporating a citrulline as a replacement for an arginine.

For the peptide E-12-D, only the arginine residue corresponding to the 8^{th} amino acid of the sequence was replaced with a citrulline during the peptide synthesis.

The reactivity of each citrullinated and non-citrullinated peptide was assayed with respect to a normal serum, to two sera from RA patients, to anti-filaggrin antibodies (AFAs) purified from a pool of 45 sera from RA patients and to anti-filaggrin antibodies purified from 12 sera from RA patients, respectively.

EXPERIMENTAL PROTOCOL:

The wells of Nunc Maxisorp microtitration plates were covered using the noncitrullinated and citrullinated peptides E-12-D and E-12-H, respectively,

diluted to a concentration of 5 µg/ml in a PBS buffer (pH: 7.4) and incubated overnight at 4°C (final volume: 100 µl/well). The wells were saturated for 30 minutes at 37°C with 200 µl/well of PBS-Tween 20 (0.05%), 2.5% gelatin. The negative control serum (normal serum) was diluted to 1/120. The anti-filaggrin antibodies were diluted in PBS-Tween 20 (0.05%)-gelatin (0.5%) (PBS TG) such that the final concentrations of anti-filaggrin autoantibodies are those indicated in the attached Table I. The negative control serum, the RA sera and the anti-filaggrin antibodies were added (final volume: 100 µl/well) and subjected to incubation for 1 hour at 37°C and overnight at 4°C. Peroxidase-labelled goat anti-human immunoglobulin gamma heavy chain antibodies (sold by the company Southern Biotechnologies) were added to each well (dilution in PBSTG: 1/2000, final volume: 100 µl/well) and subjected to incubation for 1 hour at 37°C. The revelation was carried out by adding ortho-phenylenediamine (2 mg/ml, for 10 minutes).

The results presented in the attached Table I are given as a ratio of OD at 492 nm: citrullinated peptide signal/noncitrullinated peptide signal.

These results show that, in the majority of cases, the citrullinated peptide/noncitrullinated peptide OD ratio is higher than 1, and thus illustrate the good sensitivity of the citrullinated peptides, compared to the noncitrullinated peptides, as regards their reactivity with respect to anti-filaggrin autoantibodies.

TABLE I

Peptide	Control	RA serum 1		RA serum 2			AFA pool			AFAs purified from 12 RA sera											
	serum	10*	20*	5*	10*	20*	5*	10*	20*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*
E-12-D	1.076	1.42	1.85	2.42	3.77	5.57	1.77	1.63	1.48	1.99	1.38	2.48	1.19	1.12	3.50	1.87	5.19	1.13	1.57	1.11	1.65
E-12-H	1	1.32	1.20	10.44	11.51	8.38	2.45	2.42	1.82	7.16	2.05	1.06	1.18	0.76	13.57	4.14	3.18	1.14	3.66	1.22	5.84

*: AFA concentration in μg/ml.

CLAIMS

1) Peptide comprising an epitope recognized by anti-filaggrin autoantibodies present in serum from rheumatoid arthritis patients, characterized in that said epitope comprises a tripeptide motif centred on a citrulline residue, which is specifically present on at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6.

2) Peptide according to Claim 1, characterized in that it comprises the tripeptide motif Ser-Cit-His in which Cit represents a citrulline residue.

3) Artificial antigen recognized specifically by anti-filaggrin autoantibodies present in serum from rheumatoid arthritis patients, characterized in that it comprises or consists of at least one peptide according to either of Claims 1 and 2.

4) Use of an antigen according to any one of Claims 1 to 3 for diagnosing rheumatoid arthritis *in vitro*.

5) Antigenic composition for diagnosing the presence of rheumatoid arthritis-specific autoantibodies in a biological sample, characterized in that it contains at least one antigen according to any one of Claims 1 to 3 which is optionally labelled with and/or conjugated to a carrier molecule.

6) Method for detecting rheumatoid arthritis-specific autoantibodies in a biological sample, this method being characterized in that it comprises:

- bringing said biological sample into contact with at least one antigen according to any one of Claims 1 to 3 under conditions which allow the formation of an antigen/antibody complex with the rheumatoid arthritis-specific autoantibodies possibly present;
- detecting, by any suitable means, the antigen/antibody complex possibly formed.

7) Kit for detecting rheumatoid arthritis-specific autoantibodies in a biological sample, characterized in

that it comprises at least one antigen according to any one of Claims 1 to 3, as well as suitable buffers and reagents for constituting a reaction medium which allows the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

ATTORNEY DOCKET NO.: 045636-5037

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PEPTIDE EPITOPES RECOGNIZED BY ANTI-FILAGGRIN AUTO-ANTIBODIES IN SERUM FROM RHEUMATOID ARTHRITIS PATIENTS

the specification of which:

is attached hereto; or

was filed as United States application Serial No. _____ on _____ and was amended on _____ (if applicable); or

was filed as PCT international application Number PCT/FR98/02899 on 29 December 1998 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S):

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
France	FR 97 16,673	30 December 1997	[X] Yes [ ] No
			[ ] Yes [ ] No

Combined Declaration For Patent Application and Power of Attorney - (Continued)
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5017

I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.	U.S. FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

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Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5037

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Combined Declaration For Patent Application and Power of Attorney - (Continued)
(includes Reference to PCT International Applications)

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Combined Declaration For Patent Application and Power of Attorney - (Continued)
(includes Reference to PCT International Applications)

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Listing of Inventors Continued on attached page(s) [ ] Yes [X] No

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

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